Molecular cytogenetic characterisation of a leaf-rust resistant wheat-*Thinopyrum ponticum* partial amphiploid

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**Doctoral Dissertation**

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**Abbreviations**

**BAC-FISH:** bacterial artificial chromosome fluorescence *in situ* hybridisation  
**BC1:** first backcrossed generation  
**cdc28:** cell division control 28  
**Cdk2:** cyclin-dependent kinase 2  
**cDNA:** complementary DNA  
**DAPI:** 4',6-diamidino-2-phenylindole  
**EDTA:** ethylene-diamine-tetra-acetic acid  
**EST:** expressed sequence tag  
**FL:** fraction lengths  
**FISH:** fluorescence *in situ* hybridisation  
**GISH:** genomic *in situ* hybridisation  
**Hop1:** homeodomain protein 1  
**Ime2:** inducer of meiosis 2  
**ISH:** *in situ* hybridisation  
**ITS:** internal transcribed spacer  
**mcGISH:** multicolour GISH  
**MAS:** marker assisted selection  
**MBC:** map based cloning  
**PBS:** phosphate buffered saline  
**Ph1 gene:** pairing homoeologous gene  
**RIL:** recombinant inbred line  
**SNP:** single nucleotide polymorphism  
**SSC:** saline-sodium citrate  
**SSR:** simple sequence repeats  
**TNB:** TRIS- Na blocking buffer
1. Introduction

Due to modern wheat breeding efforts, which resulted in the development of new high-yielding wheat cultivars, global crop production has increased in recent decades. The introduction of modern wheat varieties has been a major factor in the rapid growth of yield and production in many developing regions. However, modern high-yielding cultivars have resulted in the erosion of the genetic diversity of wheat (*Triticum aestivum* L.) which, combined with climate change effects, has made it increasingly vulnerable to biotic and abiotic stresses. There is an increasing demand to transfer desirable genes into bread wheat through genetic manipulation techniques (Tamás et al 2009) or classical methods such as including ancient wheat varieties in modern breeding programs (Bedő et al. 1998). Wild wheat relatives, including wheat progenitors and other closely related species, possess a wide range of genetic diversity which could widen the genetic base of cultivated wheat. The agronomically useful traits of wild species such as resistance against pests and diseases and tolerance of abiotic stresses can be incorporated into the wheat genome via wide hybridisation. The production of wide hybrids of wheat would provide a rich source of genes for plant breeders, making it possible to develop more tolerant or resistant high-yielding wheat cultivars in the future. However, the production of wide hybrids alone is not sufficient to improve the biotic or abiotic stress resistance of wheat. To achieve effective gene transfer from a related species to wheat it is essential to understand the cytogenetic background of hybrid plants carrying the desired traits. With the improvement of GISH techniques the detection and identification of alien chromatin in a wheat background has become possible. Moreover, multicolour GISH enables the discrimination of two or more closely related genomes within a polyploid species. The discrimination of the A, B and D genomes of hexaploid wheat provides information about the identity of the genomes involved in intergenomic rearrangements.

Molecular approaches, such as the application of molecular markers, offer more precise genome analysis, as they have been successfully used to identify alien introgressions that cannot be identified using *in situ* hybridisation (Nagy et al 2002). The availability of microsatellite markers, that are highly polymorphic and codominant opens up the possibility to construct molecular maps covering whole genomes, including genetic and physical maps. Physical maps reveal the precise chromosomal location of markers associated with agronomically important traits.
The line BE-1 was a result of the first Hungarian wheat–Thinopyrum ponticum hybridisation program carried out by T. Rajháthy and Á. Kiss in Martonvásár in 1953 (Belea 1964). The objective of these early crosses was to develop new, disease-resistant, high protein genetic material. BE-1 was selected by D. Szalay in 1955 from the F$_3$ generation of the cross for its high protein content and resistance to leaf rust (*Puccinia triticina*) and powdery mildew (*Blumeria graminis f. sp. tritici*) (Szalay 1979). Besides its favourable characteristics, the line BE-1 was found to be stable and highly fertile, and was thus considered suitable genetic material for increasing the protein content and transferring disease resistance into wheat. Although its general appearance was intermediate between wheat and *Th. ponticum*, in the absence of appropriate cytogenetic techniques alien chromatin could not be detected in the line.

The present study aimed to use GISH, with genomic probes originating from diploid and decaploid Thinopyrum and Pseudoroegneria species, in order to detect alien chromatin in line BE-1 and to determine its precise chromosome composition. In addition, it was planned to use FISH with various repetitive DNA probes in order to identify the wheat chromosomes and any intergenomic rearrangements. However, minor rearrangements, or the rearrangement of chromosomal regions without a characteristic FISH pattern, cannot be identified by FISH. The availability of molecular markers makes it possible to overcome such difficulties. Thus, further analysis of BE-1 was planned by exploiting chromosome-specific SSR markers, with the objective of precisely defining minor intergenomic rearrangements.

These approaches answer several important questions involving the line BE-1, such as the cause of the fertility, the quantity of alien and wheat genomes present and the frequency of intergenomic translocations. It is also possible to identify which genomes are likely to be involved in rearrangements. The cytogenetic characterisation of BE-1 would facilitate the selection of the most advantageous cytogenetic procedure for the introgression of the desired traits into cultivated wheat.
1.1 Objectives

The aims of the present study were:

- to improve the GISH technique to make it a convenient method for the routine
detection of *Thinopyrum* chromatin in wheat-*Thinopyrum* hybrids, thus facilitating the
incorporation of desired traits into the wheat genome

- to validate the hybrid status of the BE-1 partial amphiploid line

- to determine the number of chromosomes originating from the *Th. ponticum* parent

- to identify all the wheat chromosomes present in order to reveal substitutions or
additions in the wheat genome, if any

- to detect and identify wheat-*Thinopyrum* translocations and intergenomic
rearrangements among the A, B and D genomes of wheat

- to use the detected translocation chromosomes in the physical mapping of molecular
markers

- to start a backcross program using the partial amphiploid BE-1 in order to transfer its
leaf rust resistance to wheat
1.2 State of knowledge

1.2.1 Classification and phylogeny within the *Triticeae*

The tribe *Triticeae* contains more than 500 annual and perennial grasses including crops of major economic importance such as wheat, barley and rye (Löve 1984, the NCBI taxonomy homepage: [http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi](http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi)).

In terms of genome composition the *Triticeae* represent a diverse group with several distinct genomes and ploidy levels ranging from 2x to 10x. Classical cytogenetic studies have revealed that despite the large scale of genome diversity, diploid genomes of the *Triticeae* share a considerable genetic similarity (homoeology). According to the degree of genome similarities distinct genomes of *Triticeae* were assigned using capital-letter symbols (Kihara and Lilienfeld 1932, Wang et al. 1996).

The frequent occurrence of polyploids carrying several distinct genomes complicates the understanding of phylogenetic relationships within the whole tribe. Polyploids were formed by whole genome duplication (autopolyploids, e. g. *Hordeum bulbosum* L.) or by interspecific hybridisation followed by chromosome doubling (allopolyploids, e. g. hexaploid wheat). The increased genetic diversity and the major genetic and epigenetic changes (non-random elimination of coding and non-coding DNA, gene silencing via DNA methylation) which follow genome duplication make polyploidy a major force in plant evolution (Hegarty and Hiscock 2008). There is strong evidence that many plant species traditionally considered to be diploid (such as *Arabidopsis* or maize) have undergone at least one cycle of polyploidisation events during their evolution (Simillion et al. 2002, Blanc and Wolfe 2004, Rabinowicz and Bennetzen 2006).

Early phylogenetic studies based on morphological characteristics (Kellogg 1989), substitution compensation analysis (Sears 1966) and the chromosome pairing pattern in meiosis (Lilienfeld 1951, Kihara 1954) led to the construction of phylogenetic trees, but it was later questioned whether these methods were effective means for describing phylogenetic relationships (Seberg and Petersen 1998). The development of molecular genetic tools such as restriction site maps of the chloroplast genome, sequences of chloroplast genes and nuclear sequence data (5S RNA “short spacer array”, 5S RNA “long spacer array”, ITS sequences, EST sequencing, etc.) allowed a more precise reconstruction of the evolutionary relationships between the diploids (Kellogg et al. 1996, Paterson 2006).
Based on the available phylogenetic evidence it was concluded that diploid species of the *Triticeae* were derived from a common ancestral genome by divergent evolution ~4 million years ago (Huang et al. 2002; Petersen et al. 2006). The common ancestor gave rise to the lineages *Critesion*, *Hordeum* and *Psathyrostachys* and subsequent evolution led to line *Secale*. The next diverged lineages included two main clades of the tribe, one containing *Pseudoroegneria*, *Agropyron* and *Australopyrum* and the other including the sister group *Aegilops/Thinopyrum/Lophopyrum/Crithopsis* (the “ACLT clade”) (Kellogg et al. 1996). It was shown that the diploid *T. monococcum* (L.) was a result of an introgression occurring between a member of the ACLT clade and an early-diverging member of the tribe (Fig. 1).

Cytogenetic data suggest that the ACLT clade is monophyletic, though some genomic distance was detected between the genera.

The similarity (homoeology) between the wheat genomes and the genomes carried by its wild relatives opens up the possibility for interspecific hybridisation which, together with genetic engineering techniques, represents one of the most effective means for transferring new genetic material into wheat (Gale and Miller 1987; Feldman 1988; Jauhar 2006).

**Figure 1.** Evolution of the diploid *Triticeae* (Kellogg et al. 1996)
1.2.1.1 Hexaploid wheat (*Triticum aestivum* L.)

Wheat carries three homoeologous genomes, each having seven pairs of chromosomes. Its evolutionary history is the most outstanding example of successful polyploidisation within the plant kingdom. Hexaploid wheat derived through spontaneous hybridisation ~10,000 years ago between a domesticated form of tetraploid wild emmer, *T. turgidum ssp. dicoccon* (2n=4x=28, AABB) and the diploid goatgrass *Aegilops tauschii ssp. strangulata* (2n=2x=14, DD) (Fig. 2) (Kihara 1944, McFadden and Sears 1946, Dvorak et al. 1998). Using sequence analysis of the wheat ABCT-1 gene, Dvorak et al. (2006) discovered the existence of gene flow from wild tetraploid wheat (*T. turgidum ssp. dicoccoides*) to hexaploid wheat, thus demonstrating that wild tetraploid wheat also participated in the evolution of cultivated wheat.

The wild emmer (*T. turgidum ssp. dicoccoides*) arose ~0.5 million years ago from hybridisation between the wild diploid *T. urartu* (2n=2x=14, AA) and two different types of another wild diploid which were the donors of the B genome (Fig. 2) (Dvorak et al. 1988, Huang et al. 2002). The origin of the B genome remains controversial. Early analysis based on chromosome pairing in meiosis concluded that the source of the B genome was *Agropyron triticeum* (McFadden and Sears 1944), but recent studies suggested that the diploid ancestor of the B genome was an unknown species from the *Sitopsis* section, closely related to *Aegilops speltoides* (2n=14, SS) (Riley et al. 1958, Tsunewaki and Oghihara 1983, Huang et al. 2002). Molecular phylogenetic analysis based on the sequence comparison of coding and non-coding DNA regions updated the phylogeny data and led to the conclusion that the relationship between the B genome and the S genome of *Ae. speltoides* is not as close as was reported earlier. The B-genome donor of hexaploid wheat thus remains to be identified (Salse et al. 2008).

In some cases the genetic composition of polyploids represents a handicap compared to the diploids. The presence of several distinct genomes results in a higher complexity of chromosome pairing during meiosis. Such changes in meiotic behaviour may cause the deletion or addition of chromosomes, which is manifested in lower fertility at the phenotypic level. However, hexaploid wheat is a stabilised allopolyhaploid, as the pairing between homologous chromosomes is under genetic control. The presence of the *pairing homoeologous 1 (Ph1)* gene ensures diploid-like meiotic behaviour, so chromosome pairing is restricted to homologues, whereas homoeologous chromosome pairing is largely restricted. The Ph1 locus has been mapped to the long arm of chromosome 5B (Sears 1976).
Figure 2. Evolution of hexaploid wheat

Two mutant lines (ph1b, ph1c) carrying a deletion of the Ph1 locus have been produced, in which the occurrence of homoeologous pairing and hence multivalent formation at metaphase I was demonstrated (Sears 1977, Giorgi 1978). The original deletion in ph1b involved 70 Mb containing over 200 genes, but more recent studies have pinpointed the position of the Ph1 in a 3 Mb region containing less than seven genes (Gill et al. 1993, Moore 2002, Al-Kaff et al. 2008). It has been revealed that the Ph1 locus has a crucial effect on homologous chromosome recognition by ensuring synchronized chromatin remodelling during the premeiotic S phase (Colas et al. 2008). On the other hand, Ph1 has been shown to be related to the Cdk2 locus in mammals and the Ime2 gene in yeast. These kinases, as well as the related kinase cdc28, have been shown to control premeiotic replication and the formation of double strand break, and to reduce the level of the synapsis protein Hop1, which is essential for correct associations during synapsis (Griffiths et al. 2006, Moore and Shaw 2009). Such homology among the genes controlling premeiotic and meiotic procedures suggests conserved basic mechanisms of homologue recognition within plants, yeasts and mammals.
Species belonging to the present *Thinopyrum* genus (from Greek: thyno=shore, pyros=wheat) were originally classified in the *Agropyron* [(L) Gaertner] complex based on morphological characteristics. Accordingly, the *Agropyron* group included almost all the perennials having one spikelet per node, which resulted in a very large, heterogeneous genus containing more than 100 species (Table 1.) (Hitchcock 1951). This definition of the genus was not particularly useful. Nevski (1933), a taxonomist from the former Soviet Union, was the first who, in addition to morphological data, applied a cytological approach in the taxonomy of the *Triticeae*. He divided *Agropyron* into four relatively homogeneous genera: *Agropyron*, *Roegneria*, *Antosachne* and *Eremopyrum* (Table 1.). Thus *Agropyron* as defined by Nevski was narrowed to a dozen species closely related to crested wheatgrass [*A. cristatum* (L.) Gaertn.]. The generic treatment published later by Löve (1980) and Dewey (1984) was essentially based on genomic data and supported the idea that taxonomies should reflect the evolutionary relationships within a group. They determined the genomic composition of a type species and brought all taxa with the same basic genome into that genus, then excluded all taxa that did not have the same basic genome or combination of genomes as the type species. Löve (1980) partitioned the *Agropyron* genus into genera: *Agropyron*, *Pseudoroegneria*, *Thinopyrum*, *Trichopyrum* and *Lophopyrum* (Table 1.). *Agropyron* sensu Löve was limited to less than 10 species, including the type species *A. cristatum* (2n=14, PP), whose genome was designated as P. All species that contained other than the P genome were excluded from *Agropyron*.

The genome designation of *Pseudoroegneria* was S, which should not be confused with the S genome of the *Sitopsis* section. It is a small genus consisting of 11 species based on the type species *Ps. strigosa* (M. Bieb.) Löve (2n=14, SS). Löve treated *Lophopyrum* as a genus, with the genomic designation E, while Dewey placed it in the *Thinopyrum* genus with sectional status (Table 1.). Löve transferred six species from the former *Agropyron junceum* (L.) Beauv. complex into the newly designated *Thinopyrum* genus (Löve 1982). The new genus was characterised by the J genome and the type species was *Thinopyrum junceum* (L.) Löve, a hexaploid (2n=42) maritime grass. Dewey expanded *Thinopyrum* to about 20 species, including three species from the genera *Lophopyrum* sensu Löve and *Elytrigia* sensu Löve (Dewey 1984). Dewey also divided the *Thinopyrum* genus into three species complexes, each with sectional status (sections: *Thinopyrum*, *Lophopyrum*, *Trichophorae*, Table 1.).
The distinction between the *Thinopyrum* (J genome) and *Lophopyrum* (E genome) genomes was a matter of debate for decades. According to Dewey (1984) "the J genome of *Thinopyrum* and E genome of *Lophopyrum* are so close that the two genomes and the two genera should be combined." He designated both genomes as J. On the basis of the chloroplast sequence data the two taxa are indistinguishable, whereas the chromosome pairing pattern in meiosis, karyotype differences, and data on 5S DNA spacer and ITS sequences provide clear evidence that they represent different genera (Jauhar 1990, Kellogg et al. 1996).

The traditional, morphological definition of the *Agropyron* genus caused some ambiguity as both diploid and decaploid species were referred to as *Thinopyrum elongatum* (syn. *Agropyron elongatum*). Having two morphologically and genomically different species with the same name has been a source of controversy among taxonomists. Based on clear differences Dewey (1984) corrected the name of the decaploid species to *Th. ponticum*, retaining the name *Th. elongatum* for the diploid species. However, Löve placed both species in *Lophopyrum* and designated the diploid species as *Lophopyrum elongatum*, and the decaploid species as *Lophopyrum ponticum*. Later, Cauderon and Saigne (1961), and Dvorak (1981) demonstrated that the genomic composition of the decaploid is closer to *Thinopyrum sensu stricto*, so it can be designated as *Th. ponticum*.

**Table 1. Genera derived from the former Agropyron genus according to various taxonomists**

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<td><em>Trichophora</em></td>
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**Thinopyrum ponticum** (Podp.) Z.W. Liu & R. R. -C. Wang [syn Agropyron elongatum (Host) Beauvoir ssp ruthenicum Beldie]

*Thinopyrum ponticum* is a decaploid species (2n=10x=70), frequently used in wheat improvement as a donor of various disease resistance genes, in particular for leaf rust (Friebe et al. 1996) and wheat streak mosaic virus (Sebesta et al. 1972, Martin et al. 1976, Jiang et al. 1993).

The leaf rust resistance gene *Lr19* and the stem rust resistance gene *Sr25* were transferred together from *Th. ponticum* to the long arm of wheat chromosome 7D using radiation treatment (Sharma & Knott, 1966; Knott, 1968). The translocated fragment carrying genes *Lr19/Sr25* is present in wheat cultivar Agatha and measures 2.55 µm (Friebe et al. 1996). Later Sears (1973, 1977) also transferred genes *Lr19* and *Sr25* to wheat by induced homoeologous recombination. Although the translocated segment associated with *Lr19/Sr25* was transferred to Swedish (Sunnan) and Mexican (Oasis 86) wheat cultivars (McIntosh et al. 1995), it was considered unsuitable for further use in wheat improvement as it also carries an undesirable gene for yellow flour pigmentation. Other rust resistance genes have also been transferred from *Th. ponticum* to wheat, such as *Lr29* (Sears 1973, 1977), *Sr43* (Knott et al. 1977), *Lr24/Sr24* (Smith et al. 1968, Sears 1973) and *Sr26* (Knott 1968).

Efforts to reveal the genomic composition of *Th. ponticum* have been underway for decades and various hypotheses have been proposed. Classical cytogenetic analysis based on the chromosome pairing behaviour during meiosis (Cauderon 1966; Muramatsu 1990) suggested that *Th. ponticum* is an autopolyploid species and its genomic formula was designated as J\(_1\)J\(_2\)J\(_3\)J\(_4\)J\(_5\).

GISH analysis demonstrated that E\(^b\) (=J genome) from *Th. bessarabicum* and S\(^i\) genome from *Pseudoroegneria spicata* were actually the two basic genomes of *Th. ponticum* (Zhang et al. 1996). In addition, GISH using S genomic DNA, revealed that the genomic composition of *Th. ponticum* was JJ'JJJ. The J genome is homologous to the J genome of the diploid *Thinopyrum bessarabicum*, whereas the J' genome is a modified J genome of unknown origin characterised by the presence of an S genome-specific hybridisation signal near the centromere (Chen et al. 1998c).
1.2.2 Wide hybridisation

1.2.2.1 Historical background

Efforts to cross hexaploid wheat with related species have been made for centuries. According to Ciferri (1955) the first interspecific wheat hybridisation experiments were performed by Barelle in 1806 by pollinating *Triticum polonicum* flowers with *Triticum compositum* pollen, resulting in fertile grains. Wilson (1876) was the first to use *Agropyron* and rye pollen to fertilise hexaploid wheat in his greenhouse in Scotland, but the wheat-rye hybrid was almost completely sterile. The first fertile *Triticale* was found 15 years later by Rimpau (1891), who obtained seeds on a natural wheat-rye hybrid. The first hybridisation between wheat and barley was reported in 1904 by Farrer, although the authenticity of the hybrid plant was later questioned (Shepherd and Islam 1981). Farrer was unable to confirm the success of the cross as the wheat-barley hybrids and their progeny did not unequivocally manifest any barley characters. *Aegilops* species were also crossed with wheat in the early 1920’s and 1930’s (Kihara 1937), which led to the discovery of the genomic relationships within the genus.

An intensive hybridisation program involving annual wheat and species from the former *Agropyron* genus was successfully carried out from the early 1930’s by Tsitsin in the former Soviet Union (Tsitsin 1960, 1975). The objective of these early programs was to transfer disease resistance or the perennial habit of *Agropyron* to wheat.

The first wide-ranging interspecific hybridisation project in Hungary was commenced in 1950 in Martonvásár (Lelley and Rajháthy 1955) and in 1971 in the Institute of Genetics of the Szeged Biological Centre. These crosses aimed to develop high-protein disease-resistant genetic material for future wheat breeding programs.

Though efforts to cross hexaploid wheat with *Agropyron* were made in the 1940’s by Árpád Kiss in Mosonmagyaróvár, the first successful wheat-*Agropyron* hybrids were produced in 1953 by Árpád Kiss and Tibor Rajháthy in Martonvásár (Belea 1964). F₁ hybrids were obtained from the combinations *T. aestivum × Th. intermedium* and *T. aestivum × Th. ponticum*. The old Hungarian bread wheat (*T. aestivum*) cultivars F 481 and Bánkúti 1201 were used as hexaploid wheat progenitors. The partial amphiploid line BE-1 was the result of this hybridization program. It was selected by Szalay Dezső in 1955 from the F₃ progenies of the Bánkúti 1201×*Th. ponticum* hybrid, on the basis of favourable characters such as high protein content and disease resistance (Szalay 1968). The advantage of BE-1 in wheat
improvement is not due only to its favourable traits, but also to the fact that it is stable and highly fertile and can thus be used effectively for increasing the protein content and transferring disease resistance into wheat.

1.2.2.1 Technical background

Early interspecific hybridisation programs were carried out using simple methods such as emasculation and pollination, which in some cross combinations failed to produce the desired hybrids. During this period many species remained inaccessible to wheat breeding programs. With the improvement of cytogenetic techniques the production of complex hybrids became feasible, making it possible to introduce desired traits from a wide range of species into the hexaploid wheat (Kruse 1974, Islam et al. 1978, Wang 1989, Molnár-Láng and Sutka 1994). The discovery of colchicine as an amitotic agent (Eigisti 1940) and the application of embryo-rescue techniques (Sharma and Gill 1983) extended the range of species involved in interspecific hybridisation.

Large differences in crossability between different species of Triticeae and even between different wheat varieties suggested the existence of crossability genes. The dominant alleles of these genes – \(Kr1, Kr2\) (Riley and Chapman 1967) \(Kr3\) (Krowlow 1970) and \(Kr4\) (Zheng et al. 1992) – were found to reduce crossability with rye and also to influence crossability with other related species in the Triticeae (Snape et al. 1979, Falk and Kasha 1981, Molnár-Láng and Sutka 1989).

The dominant alleles, present in most European wheat varieties, suppress the crossability with rye, whereas genotypes from China, Japan, East Siberia and Iran carry recessive alleles, resulting in high crossability (Zeven 1987, Ma et al. 1996). Chinese Spring, a strain of a Chinese landrace, was selected as a model cultivar for genetic research on wheat particularly for its high crossability with rye.

The loci \(Kr1\) and \(Kr2\) were mapped to chromosomes 5B and 5A, respectively (Sitch et al. 1985), while \(Kr3\) was mapped to 5D (Krolof 1970). Further analysis of Chinese landraces of wheat led to the discovery of a new locus controlling crossability named \(Kr4\), which was mapped to chromosome 1A and was considered to have a stronger effect than \(Kr2\) or \(Kr3\) (Zheng et al. 1992). \(Kr1\), expressed in the inflorescence of the plant, reduces crossability by blocking the entry of pollen tubes into the micropyle of the maternal tissue (Fedak and Jui 1982). It was demonstrated that two other genes, \(Vrn1\), related to vernalisation (Galiba et al. 1995), and \(Ph1\), controlling homologous chromosome pairing during meiosis, are located
relatively close to *Kr1* on a gene-rich cluster on 5BL (Gill et al. 1996a, Griffiths et al. 2006). However, not only was a physical relationship detected between these genes, but there is also evidence that all three genes may have arisen from a single ancestral gene by neofunctionalisation (Tixier et al. 1998). Efforts to reveal the molecular function of the *Kr1* gene were made using a 5B chromosome RIL population, and putative candidate genes were identified, which are mainly involved in stress signals and photoinduction (Manickavelu et al. 2009).

The transfer of recessive crossability genes into modern wheat cultivars is feasible and could facilitate the exploitation of related species in wheat improvement (Molnár-Láng et al. 1996).
1.2.2.3 Wheat-\textit{Thinopyrum} amphiploids

With the improvement of crossing techniques the production of interspecific hybrids of wheat became feasible. However, due to their unbalanced genetic background \(F_1\) hybrids are generally almost completely sterile. The discovery that colchicine treatment induces polyploidisation (Eigisti 1940), thus allowing the fertility of \(F_1\) hybrids to be restored, led to the production of a number of wheat-alien amphiploids. Colchicine treatment inhibits the formation of spindle fibers and arrests cell division in metaphase, which resulting in chromosome doubling. Amphiploids can also be synthesized naturally by the formation of unreduced gametes (Sharma et al. 1987). During the stabilisation of the amphiploid, the high number of homoeologous genomes present may result in substitutions and/or deletions involving the wheat or the alien genomes and the chromosome number usually stabilizes at 56 (Chen et al. 1998b, Fedak et al. 2000, Oliver et al. 2006). Stable amphiploids generally carry a complete set of the wheat genomes (AABBDD) and one set of the alien genome (XX) and show ploidy stabilisation at the 8x level \((2n = 56)\). However, in a few cases the substitution of wheat chromosomes was observed (Fedak and Han 2005). Substitutions or deletions usually involve homoeologous chromosomes, as the effect of a missing chromosome pair can be compensated by its homoeologue (Knott 1968, Li et al. 2004).

Amphiploids, being stable and highly fertile, represent an important intermediate step in wheat breeding programs (Gale and Miller 1987, Jiang et al. 1994, Ellneskog-Staam and Merker 2002). Moreover, they allow the reliable analysis of the effects of alien genes in the genetic background of wheat. Several \textit{Th. ponticum}–wheat amphiploid lines have been obtained, such as Agrotana, OK7211542, PWM706, PWMIII and PWM 209, which were proved to carry many agronomically useful traits (resistance to wheat streak mosaic virus, barley yellow dwarf virus, common root rot, \textit{Fusarium} head blight, tan spot and \textit{Stagonospora nodorum}) originating from the \textit{Th. ponticum} progenitor. These have been analysed and exploited as alien sources of disease resistance in wheat improvement (Chen et al. 1998a, 1998b; Thomas et al. 1998; Fedak et al. 2000; Li et al. 2004; Oliver et al. 2006).

BE-1, selected by Szalay D. (1979), is a wheat–\textit{Thinopyrum ponticum} partial amphiploid with 56 chromosomes, having high protein content and resistance to leaf rust and powdery mildew (Szalay 1979). Being highly fertile, this genetic material could be a potential source for wheat improvement.
Molecular analysis of interspecific hybrids

1.2.3.1 In situ hybridisation

After the first wide hybridisation experiments the necessity of an efficient hybrid validation method became apparent. The authenticity of early hybrids was investigated using morphological characteristics and subsequently by chromosome counting in the mitotic metaphase of the presumed hybrids (Mujeeb-Kazi and Miranda, 1985). More information was provided about the individual identity of the chromosomes, and thus about the hybrid identity, by the introduction of chromosome banding techniques (Gill and Kimber 1974, Hadlaczky and Belea 1975). Nevertheless, a number of species manifest a lack of polymorphism in point of their banding pattern, which renders hybrid validation more difficult. The establishment of ISH technique by Rayburn and Gill (1985) and later the GISH technique by Schwarzacher et al. (1989) for plants opened up the possibility to visualise relatively small introgressions of alien chromatin in interspecific hybrids (Le et al. 1989; Molnár-Láng et al. 2000; Cai et al. 2001). GISH based on hybridisation in situ with labelled total genomic DNA of the introgressed species on mitotic metaphase preparations of hybrid plants, allows the discrimination of alien chromosomes in a wheat background.

The advent of mcGISH techniques provided the opportunity for more precise genome analysis. This method is suitable for the simultaneous discrimination of several genomes in allopolyploid plants. Multicolour GISH using several different genomic probes is a possibility for simultaneously visualising two or more genomes in a polyploid species. Mukai et al. (1993) simultaneously visualized the A, B and D genomes of hexaploid wheat using A and D genomic probes. Han et al. (2003) used multicolour GISH to analyse wheat–Thinopyrum intermedium derivatives and detected intergenomic rearrangements involving Th. intermedium chromosomes and the A and B genomes of wheat. Molnár et al. (2009) detected irradiation-induced translocations in wheat-Aegilops biuncialis amphiploids among the U and M chromosomes of Ae. biuncialis and chromosomes of hexaploid wheat. Zhang et al. (2004) successfully performed BAC-FISH to paint the A, B and D genomes of wheat, and detected intergenomic translocations involving the A genome and the A and D genomes of hexaploid wheat.

The FISH technique involving the hybridisation of repetitive DNA clones reveal the chromosomal distribution of these sequences. The visualisation of major repeat clusters results in a specific banding pattern which makes chromosome identification possible
(Bedbrook et al. 1980; Rayburn and Gill 1987; Mukai et al. 1993; Pedersen and Langridge 1997) and is able to reveal intergenomic chromosome rearrangements in a polyploid species (Linc et al. 1999). The combination of the mcGISH technique with sequential FISH on wheat alien hybrids enables chromosomes belonging to different genomes to be detected and identified and intergenomic rearrangements involving several genomes of a polyploid species to be visualized (Sánchez-Morán et al. 1998, Nagy et al. 2002, Wang et al. 2005).

When using FISH on mitotic metaphase chromosomes, the shortest DNA sequence that can be detected unambiguously (=detection sensitivity) is 4-5 Mb and the smallest physical distance between target sequences that can be resolved with a fluorescence microscope (=spatial resolution) is 100kb. Higher spatial resolution (0.12 Mb) and detection sensitivity (50.0 kb) can be achieved by using meiotic prophase preparations, in particular pachytene chromosome preparations for in situ hybridisation where, due to chromatin decondensation, chromosomes are 10- to 40-fold longer than in mitotic metaphase (resolution and sensitivity: 4-5 Mb) (de Jong et al. 1999). Spatial resolution can be increased even more (1 kb) by using FISH on extended DNA fibers (Valarik et al. 2004).

The identification of the introgressed chromatin and its clear distinction from that of the recipient species by means of ISH techniques makes it possible to follow the behaviour of alien chromosomes or chromosome segments in both the mitotic and meiotic phases, and to elucidate in detail genomic rearrangements such as deletions or translocations. In addition, intergenomic rearrangements visualized by GISH or mcGISH and identified by FISH using repetitive DNA sequences represent excellent genetic materials for cytogenetically based physical mapping (Biagetti et al. 1999).
1.2.3.1 Physical mapping

The physical localisation of genes or molecular markers to the 17 Gb wheat genome has opened up new possibilities in the understanding of many aspects of its structure, function, and evolution. An understanding of the molecular basis of the phenotypic traits is indispensable for accelerating breeding and wheat improvement.

The localisation of molecular markers, including SSRs, ESTs or SNPs, to a precise chromosomal region and subsequently the construction of cytogenetically based physical maps are essential for the high quality assembly of DNA sequences from genome sequencing projects including BAC-by-BAC and whole-genome shotgun sequencing (Dolezel et al. 2007). The complete physical mapping of the largest wheat chromosome (3B) was performed using a chromosome-specific BAC library originating from sorted 3B wheat chromosomes of the Chinese Spring wheat cultivar assembled into 1036 contigs that were anchored with 1443 molecular markers (Paux et al. 2008).

With the availability of cytogenetically-based physical maps it also became feasible to answer important questions associated with genome organisation, evolution and the comparative genetic analysis of species (Faris et al. 2000, Sandhu and Gill 2002, Duran et al. 2009). This is well illustrated by the fact that the localisation of specific DNA sequences to a precise chromosomal region (physical mapping) revealed a high proportion (more than 90%) of retrotransposon-like repetitive sequences in the wheat genome. By mapping cDNA probes, regions containing expressed genes were also identified and were demonstrated to cover less than 10% of the wheat genomes. Moreover, physical and high density linkage mapping revealed that the majority of the wheat genes are present in clusters (gene-rich regions) that generally occur more frequently in distal parts of the chromosomes. Comparative analysis of genetic and physical maps revealed that recombination events are likely to occur in gene-rich regions termed recombination hotspots, while cross-overs are strongly suppressed in the heterochromatic regions characteristic of the centromeres (Gill and Gill 1994, Gill et al. 1996a, Gill et al. 1996b). Recombination is initiated by meiosis-specific double strand breaks which occur in highly decondensed gene-rich regions showing hypersensitivity to nucleases.

Genetic maps are based on the recombination frequency between markers, measured in centimorgans (cM). For this reason, genetic mapping provides only an estimate of the physical distances, so it cannot be used to measure physical distances. Physical maps, using the translocation or deletion breakpoints of aneuploid lines as a physical landmark, can allocate a molecular marker to a precise chromosomal region and distances can be measured.
in kilobase-pairs (Endo and Gill 1996). “By integrating genetic maps with hybridisation-based physical maps, resolution can be improved from centiMorgan scale to kilobase scale” (Paterson et al. 2004).

Aneuploid stocks of common wheat have been used successfully in genome mapping studies for the localisation of genes or DNA markers on chromosomes or chromosome arms (Röder et al. 1998b, Qi et al. 2003, Sourdille et al. 2004, Song et al. 2005) The production of new aneuploid wheat lines (nulli-tetrasomic, ditelosomic, deletion lines) is considered to be important for the cytogenetically-based physical mapping of the PCR-based molecular markers.

In order to create genetic or physical maps the isolation of informative DNA markers is required. It has been demonstrated that microsatellite DNA markers based on the PCR technique are highly polymorphic and codominant, making them ideal for molecular mapping analysis. Several laboratories have developed microsatellite markers, which were investigated in various genetic and physical mapping studies (Xgwm, Xgdm: Röder et al. 1998a, 1998b, Pestsova 2000; Xbarc: Song et al. 2002, Xcfa, Xcfd: Sourdille et al. 2004).

Finally, molecular mapping techniques are of great interest in modern plant breeding programs, opening up the possibility for marker-assisted selection (MAS) and map-based cloning (MBC) (Keller et al. 2005, Varshney et al. 2006). The identification and localisation of molecular markers associated to specific traits enables the selection of traits difficult to trace at the seedling stage, thus speeding up the breeding process.
2. Materials and methods

2.1 Plant material

Table 2. The plant genotypes used in the present study

<table>
<thead>
<tr>
<th>Plant species name</th>
<th>Author</th>
<th>Ploidy and chromosome No.</th>
<th>Genomic composition</th>
<th>Accession No</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BE-1</strong> <em>(T. aestivum – Th. ponticum partial amphiploid line)</em></td>
<td></td>
<td>2n = 5x = 56</td>
<td>AA BB DD JJ’</td>
<td></td>
</tr>
<tr>
<td><strong>Triticum urartu</strong></td>
<td>Thum.</td>
<td>2n = 2x = 14</td>
<td>AA</td>
<td>MVGB 115</td>
</tr>
<tr>
<td><strong>Aegilops bicornis</strong></td>
<td>Forsk.</td>
<td>2n = 2x = 14</td>
<td>SS (= BB)</td>
<td>MVGB 603</td>
</tr>
<tr>
<td><strong>Aegilops tauschii</strong></td>
<td>Coss.</td>
<td>2n = 2x = 14</td>
<td>DD</td>
<td>MVGB 363</td>
</tr>
<tr>
<td><strong>Triticum durum</strong></td>
<td>Desf.</td>
<td>2n = 2x = 28</td>
<td>AABB</td>
<td>MVGB 659</td>
</tr>
<tr>
<td><strong>T. aestivum</strong> cv. Bánkúti</td>
<td>Linné</td>
<td>2n = 6x = 42</td>
<td>AABBDD</td>
<td></td>
</tr>
<tr>
<td>cv. Mv Suba</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cv. Chinese Spring ph mutant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mv9kr1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Elytrigia elongata</strong></td>
<td>(Host) Nevski</td>
<td>2n = 2x = 14</td>
<td>EE</td>
<td>NPGS. PI 531717</td>
</tr>
<tr>
<td><strong>Thinopyrum bessarubicum</strong></td>
<td>Savul. Rayss</td>
<td>2n = 2x = 14</td>
<td>JJ</td>
<td>NPGS PI 531710</td>
</tr>
<tr>
<td><strong>Pseudoroegneria strigosa</strong> subsp. aegilopoides</td>
<td>(Drobow) A. Löve</td>
<td>2n = 2x = 14</td>
<td>SS</td>
<td>NPGS PI 531754</td>
</tr>
<tr>
<td><strong>Thinopyrum ponticum</strong></td>
<td>(Podp.) Z.W. Liu &amp; R. R. -C. Wang</td>
<td>2n = 10x = 70</td>
<td>JJJJ’J’</td>
<td>NPGS PI 340066</td>
</tr>
</tbody>
</table>
Line BE-1 was produced by crossing hexaploid wheat (*T. aestivum* cv. Bánkúti) with the decaploid species *Th. ponticum* (Szalay 1979). Resistant, highly fertile, stable progenies were selected from the F$_3$ generation of the cross.

### 2.2 Chromosome preparation

Root tips from germinated seedlings were collected and incubated in ice water for 24-26 hours. Cold-treated roots were transferred to fixative Carnoy I (3:1 v/v mixture of absolute ethanol and glacial acetic acid) and incubated for 5 days at 37°C, followed by staining in acetocarmine for 2 hours at room temperature (RT). Chromosome preparations were obtained using the squash method. After removing the coverslip, the slides were air-dried for 1–2 days at RT, then stored at -20°C for several weeks.

### 2.3 Probe labelling

For *in situ* hybridisation total genomic DNA was extracted from fresh young leaves following the phenol-chloroform method described by Sharp et al. (1988). The total genomic DNA of *T. urartu* and *Ae. tauschii* was labelled with digoxigenin-16-dUTP by nick translation (Dig-Nick Translation Mix, Roche), while DNA isolated from *E. elongata*, *Th. bessarabicum*, *Ps. strigosa* and *Th. ponticum* was labelled with biotin-11-dUTP by nick translation (Biotin-Nick Translation Mix, Roche).

FISH was carried out using the following repetitive sequences: Afa family (Nagaki et al. 1995), a subfamily clone of the pAs1 repetitive sequence originally cloned by Rayburn and Gill (1986) from *Aegilops squarrosa*, the rye subtelomeric heterochromatic sequence pSc119.2 (Bedbrook et al. 1980), the GAA microsatellite sequence and the 18S-5.8S-26S rDNA clone, pTa71 (Gerlach and Bedbrook 1979).

For three-color FISH, the pSc119.2 and Afa-family DNA sequences were amplified and labelled by PCR with biotin-11-dUTP (Roche) and digoxigenin-16-dUTP (Roche), respectively (Contento et al. 2005; Nagaki et al. 1995). The clone pTa71 was labelled combinatorially with 50% Biotin-11-dUTP and 50% Dig-11-dUTP. GAA satellite sequences were amplified from the genomic DNA of *Hordeum vulgare* L. and labelled using PCR with biotin-11-dUTP according to Vrana et al. (2000). Digoxigenin and biotin were detected using anti-digoxigenin-rhodamine Fab fragments (Roche) and streptavidin-FITC (Roche), respectively.
2.4 Genomic in situ hybridisation

Before hybridisation, the slides were pretreated with RNase A (Sigma, Munich) (100 mg/ml in 2× saline-sodium citrate, SSC) for 60 min at 37°C. The slides were then washed twice for 5 min each with 2×SSC (SSC: 3 M NaCl, 0.3 M sodium citrate, pH=7) and digested with a freshly made pepsin (Sigma-Aldrich) solution (1 mg/ml in 10 mM HCl, pH 7.5) at 37°C for 2-4 min, followed by a rinse in 1×PBS (PBS: 1.37 M NaCl, 14.7 mM KH$_2$PO$_4$, 78.1 mM Na$_2$HPO$_4$, 26.8 mM KCl). The slides were fixed in 4% paraformaldehyde in 1×PBS for 10 min, then rinsed in 1×PBS and dehydrated in a 70%, 90% and 100% ethanol series.

The hybridisation solution (25 μL/slides) contained 80 ng of biotinylated J genome specific probe, and 70 ng of digoxigenated D genomic probe or 70 ng of digoxigenated A genomic probe. For blocking, 3.2 μg AB-genomic DNA, or 1.6 μg B genomic DNA and 1.6 μg D genomic DNA were applied. The probes were mixed with 50% deionized formamide and 10% dextrane sulfate in 2 × SSC then denatured at 85°C for 8 min 30 sec. and chilled on ice for 5 min. The chromosomes were denatured in the presence of the denaturation solution (containing 75% deionized formamide, 15% dextrane sulfate in 2 × SSC) at 75°C for 6 min. Slides were rinsed and dehydrated in a 70%, 90% and 100% ethanol series. After drying at RT the slides were incubated in the presence of the hybridisation solution at 42°C overnight.

In order to detect biotinylated and digoxiginated sequences, the slides were incubated with 10 μg/mL streptavidin-FITC (fluorescein isothiocyanate) and 10 μg/mL anti-digoxigenin-rhodamine in TNB detection buffer for 45 min at 37°C. The slides were mounted in Vectashield antifade solution (Vector Laboratories) containing 2 μg/mL 4-6-diamino-2-phenylindole (DAPI). Fluorescent signals were visualized with a Zeiss Axioscope 2 epifluorescence microscope equipped with a filter for detecting DAPI (Zeiss, Filterset 01) and a dual band filter set (Zeiss, Filterset 24) for the observation of FITC and rhodamine signals simultaneously. Photographs were taken with a Spot CCD camera (Diagnostic Instruments, Inc., USA). The image processing was carried out using Image-Pro Plus 5.1 (Media Cybernetics, USA) software.

2.5 Fluorescence in situ hybridisation

After washing off the GISH hybridisation signals in 4 × SSC Tween at 25°C overnight, multicolour FISH was performed on the slides according to a protocol similar to
GISH, except that the hybridisation temperature was 37°C in this case. FISH was carried out by hybridizing three labelled repetitive DNA clones simultaneously (pSc119.2, the Afa-family and pTa71). Salmon sperm DNA was added for blocking. The procedure used for detection and counterstaining was the same as that described for GISH.

### 2.6 SSR marker analysis

Total genomic DNA from the wheat cultivar Bánkúti 1201, the amphiploid line BE-1 and the diploids *Th. bessarabicum* and *Pseudoroegneria strigosa subsp. aegilopoides* (NPGS accession No. PI 531754) was extracted according to Anderson et al. (1992).


The following PCR regime was used: 94 °C for 3 min, 45 cycles with 94 °C for 1 min, 50-55-60 and 65 °C (depending upon the annealing temperature of the primers) for 1 min and 72 °C for 2 min followed by a final extension step at 72 °C for 10 min.

The annealing temperatures of microsatellite primer pairs were: 50°C for gwm437, wmc94, barc172 barc192, barc1088, barc111, barc126 and barc214, 55°C for gwm350, gwm111, barc1046, barc64 and gwm282; 60°C for gdm46, gdm67, gdm130, gdm84, gwm295, wmc506, gdm150, cfa2019, gwm332, gwm63, cfa2123, cfa2257, cfa2049, gwm428, gwm37, cfd69, barc53, gdm142, barc184, gdm86 and gwm44; and 65°C for barc29.

PCR reactions were performed in an Eppendorf Mastercycler (Eppendorf-Netheler–Hinc Inc., Hamburg, Germany). The total volume of 16 µL reaction mixture contained 30 ng genomic DNA, 5 × PCR buffer, 0.45 U Taq DNA polymerase (both Promega, Madison, WI), 0.3 µM of forward and reverse primers, 200 µM dNTPs and 1.5 mM MgCl₂. The PCR products were separated on 2% agarose gels. The bands were stained using ethidium bromide and DNA was visualised using a SynGene GelDoc system (SynGene, Cambridge, England). The size of the PCR products was determined using the GeneTools gel analysis software (SynGene, Cambridge, England).
3. Results

3.1 Detection of alien chromatin in the partial amphiploid BE-1 by GISH

The presence of alien chromatin in the partial amphiploid BE-1 was revealed using biotinylated J-genome probe and wheat DNA for blocking. Among the 56 chromosomes of the amphiploid, 16 exhibited strong fluorescence, suggesting that these chromosomes arose from the *Th. ponticum* parent (Fig. 3A). The fact that only 40 wheat chromosomes could be counted indicated the absence of one wheat chromosome pair. It was observed that four of the alien chromosomes carried unlabelled regions in their pericentromeres, suggesting that they were involved in intergenomic translocations (Fig. 3B).

![Figure 3](https://via.placeholder.com/150)

**Figure 3.** A. *GISH on mitotic chromosomes of BE-1 using biotinylated J-genome probe. Alien chromosomes are visualized in green. Uncomplete cell. B. *GISH pattern of Thinopyrum chromosomes with J-genome probe. The J genome is visualised in green.*

One pair of *Th.* chromosomes (chromosome No 8) showed a weaker fluorescent signal in the centromeric region when J genomic probe was used, but this hybridisation pattern was
clearly different from that of chromosomes 3 and 7 (Fig. 3B). Short introgressions were detected in the intercalary region of both arms of *Th.* chromosome No. 4 (Fig. 3B).

In order to identify the unlabelled chromatin in the centromeric regions of the *Th.* chromosomes different genomic probes were applied. When J genomic DNA was replaced by E genomic DNA from *Elytrygia elongata*, the same hybridisation pattern was observed as for J genomic DNA (data not shown). However, differences were detected in the GISH pattern of the alien chromosomes when biotinylated S genomic DNA was used as probe. Sixteen chromosomes showed green fluorescence, but differences were detected in the signal intensity, particularly in the pericentromeric regions of some chromosomes (Fig. 4A). In the case of two chromosome pairs the signal intensity near the centromeres was clearly stronger than when probed with J genomic DNA. However, one chromosome pair still showed unlabelled centromeric and pericentromeric regions (Fig. 4B).

---

**Figure 4.** A. GISH on mitotic chromosomes of BE-1 using biotinylated S-genome probe. Alien chromosomes are visualized in green. B. GISH pattern of pericentromeric rearrangements detected in *Th.* chromosomes with S-genome probe. The S genome is visualised in green.
When *Th. ponticum* probe was applied for GISH, 16 chromosomes were uniformly labelled except one pair which showed no fluorescent signal at the pericentromeres (Fig. 5A, B).

**Figure 5.** A. GISH on mitotic chromosomes of BE-1 using biotinylated *Th. ponticum* probe. Alien chromosomes are visualized in green. B. GISH pattern of a pericentromeric rearrangement involving one of the *Th. ponticum* DNA is visualised in green.
3.2 Genome differentiation of BE-1 by multicolour GISH

In order to define more precisely the cytogenetic background of BE-1, two genomes were painted simultaneously with different fluorochromes resulting in three-color GISH (red, green and brown=unlabelled) which allowed the genomic composition of the partial amphiploid to be reconstructed more accurately.

By probing with biotinylated J genomic DNA and digoxigenated A genomic DNA and blocking with B and D genomic DNA, 16 *Th. ponticum* chromosomes, 14 A genome chromosomes and 26 unlabelled chromosomes were detected, indicating that BE-1 carries a complete set of A genome chromosomes, while one pair of wheat chromosomes was substituted by a pair of alien chromosomes (Fig. 6A, B).

**Figure 6.** A. Multicolour genomic in situ hybridisation on mitotic chromosomes of BE-1 using J and A genome probes. The J genome is visualised in green and the A genome in red, while B and D genome chromatin is brown (unlabelled). The 7A translocation chromosomes are indicated with arrows. The well-known 4AL translocation is marked with an asterisk. B. Fluorescent in situ hybridisation on mitotic chromosomes of BE-1 using Afa-family (red), pSc119.2 (green) and pTa71 (yellow) repetitive DNA probes.

Four of the 16 *Th. ponticum* chromosomes (later identified by FISH as two pairs) showed no green fluorescent signal near the centromeres (chromosomes TH 3 and TH 7 on Fig. 6B); however, they exhibited a faint red signal in the same region (chromosomes TH 3 and TH 7 on Fig. 6B), though the signal was weaker than that given by the A genome.
chromosomes. This suggested that the alien genome in the centromeric region of these _Th._ chromosomes did not originate from the A genome. Chromosome TH 8 (Fig. 6A, B) showed the same weak fluorescent signal in the centromeric region as observed when using only the J genomic probe, without exhibiting any affinity to the A genomic probe. Among the 14 red-fluorescing chromosomes, 2 pairs carried a terminal unlabelled region, with FL values of $\pm 0.7$ and $\pm 0.8$, respectively, on the relevant arm, suggesting that intergenomic rearrangement had taken place in the wheat genome (Fig. 6A, B; Fig. 7A, B). These chromosomes were later identified using FISH as 4A and 7A, respectively (Fig. 6B). A translocation involving the terminal region of wheat chromosome arm 4AL has already been reported by several authors as a double translocation (Naranjo et al. 1987, 1990; Chao et al. 1989; Liu et al. 1992; Devos et al. 1995). The first translocation occurred between 5AL and 4AL during the evolution of _T. monococcum_, then at the early tetraploid stage a second rearrangement took place between 4AL and 7BL. A pericentric inversion involving the 4A chromosome was also observed, but the order of the second translocation event and that of the pericentric inversion could not be determined.

Figure 7. A. Wheat chromosome 4A using A-genome probe, with the B and D genomes for blocking. The A genome is visualised in red. The unlabelled region on the terminal region of the long arm indicates the well-known 4AL-5AL-7BL translocation. B. GISH pattern of the 7A chromosome of BE-1 using A-genome probe, with the B and D genomes for blocking. The A genome is visualised in red. The short unlabelled segment on the terminal region of the long arm indicates a new, unidentified translocation.

In further probing experiments, biotinylated J genomic DNA and digoxigenated D genomic DNA were used as probes, while AB genomic DNA isolated from _Triticum durum_ was used for blocking. This study revealed only 12 D genome chromosomes, indicating that the two wheat chromosomes missing from BE-1 belonged to the D genome (Fig. 8A). One pair of A chromosomes, which showed unlabelled regions when using the A genome probe, exhibited red fluorescence in the same region in this experiment. This suggested that D-A genomic translocations had taken place (Fig. 8A). Two pairs of _Th. ponticum_ translocation
chromosomes, which were involved in centromeric translocations and showed a faint fluorescent signal on the centromeric region when A genomic DNA was used as a probe, also gave a faint fluorescent signal when probed with D genome DNA. The other pair of *Th. ponticum* translocation chromosomes exhibited no fluorescent signals on the centromeric regions when A or D genome probe was added (Fig. 8A).

**Figure 8 A.** Multicolour genomic in situ hybridisation on mitotic chromosomes of BE-1 using biotinylated J genome and digoxigenated D genome probes. The J genome is visualised in green and the D genome in red, while the A and B genomes are brown (unlabelled). The short translocated D genome segment on chromosome 7A is indicated with an arrow. **B.** Fluorescence in situ hybridisation on mitotic chromosomes of BE-1 using Afa-family (red), pSc119.2 (green) and pTa71 (yellow) repetitive DNA probes.

### 3.3 FISH using repetitive DNA probes

The identification of wheat and *Th. ponticum* chromosomes by three-color FISH, with the simultaneous hybridisation of the repetitive DNA probes pSc 119.2 and Afa family and a 18S-5.8S-25S rDNA probe (pTa71), has been successfully employed on mitotic metaphase cells of BE-1. All the wheat chromosomes present were unequivocally identified and the FISH pattern of the *Th. ponticum* chromosomes carried by BE-1 was determined (Fig. 6B, 8B, 8B). The simultaneous *in situ* hybridisation of the repetitive DNA probes showed the complete absence of the 7D chromosome pair (7D nullisomy; Fig. 8A, B). The genomic characterisation of the *Th. ponticum* chromosomes detected in the amphiploid by GISH and
FISH was also carried out in this study. The 16 added *Th. ponticum* chromosomes could be arranged in eight pairs and were differentiated from each other by their GISH and FISH patterns (Fig. 9 A, B).

---

**Figure 9 A.** GISH pattern of the eight types of Thinopyrum chromosomes in BE-1 using J-genome probe. The J genome is visualised in green. **B.** FISH pattern of the eight types of Thinopyrum chromosomes in BE-1 using Afa-family (red), pSc119.2 (green) and pTa71 (yellow) repetitive DNA probes.

*Chromosome No. 1 (Th. ponticum)*

The longest added chromosome pair was metacentric and showed a uniform green signal along its entire length when biotinylated total J genomic DNA was added as probe, indicating that the chromosome belonged to the J genome. It exhibited a faint dispersed Afa-family signal on the telomeric region of each arm (No. 1 on Fig. 9A, B).

*Chromosome No. 2*

The second chromosome pair, a submetacentric chromosome, belonged to the J genome and carried a strong Afa family signal on the terminal region of its short arm (No. 2 on Fig. 9A, B).
Chromosome No. 3

The third chromosome pair was submetacentric and labelled by the biotinylated J genome probe only in the terminal regions, while the centromeric region remained unlabelled. The fraction lengths were ±0.35 and ±0.26. When digoxigenated A or D genomic DNA was added, the centromeric region showed a faint fluorescent signal. The intensity of the signal was weaker than that on the A or D genome chromosomes, but stronger than that observed for the telomeric region of the same chromosome. This chromosome exhibited no FISH signal with the four repetitive DNA clones used (No. 3 on Fig. 9A, B).

Chromosome No. 4

The fourth chromosome pair, also a submetacentric chromosome, had a narrow unlabelled band in the intercalary region of each arm and no FISH signal (No. 4 on Fig. 9A, B).

Chromosome No. 5

The fifth was an acrocentric chromosome with a pTa71 signal on the short arm and an Afa signal on the subtelomere of the long arm (No. 5 on Fig. 9A, B).

Chromosome No. 6

The sixth was an acrocentric chromosome of the J genome type with a pTa71 signal on the distal region of the short arm and a pSc119.2 signal on the terminal region of the long arm (No. 6 on Fig. 9A, B).

Chromosome No. 7

The seventh chromosome was submetacentric and carried a translocation in the centromeric region with fraction lengths of ±0.48 and ±0.41. The A and D genome probes hybridized weakly to the centromeric region, as seen for the second largest *Th. ponticum* chromosome (chr. No. 2). It carried a pSc119.2 signal on the end of the short arm and an Afa signal on the subtelomeric region. On the long arm, two other Afa family signals were observed, one close to the centromere (near to the translocation breakpoint) and the other close to the telomere. It was the only added chromosome that had a strong GAA signal near the centromeric region (No. 7 on Fig. 9A, B).

Chromosome No. 8

The eighth and smallest chromosome was submetacentric and strongly labelled by the J genome probe at the telomeres, but was only partly labelled close to the centromere. The centromeric region was unlabelled when using the A or D genome probes. A pSc119.2 signal was observed in the terminal region of the short arm (No. 8 on Fig 9A, B).
3.4 SSR marker analysis: the 7A.7D translocation

Multicolour genomic in situ hybridisation using J and A genomic probes revealed two pairs of A-genome chromosomes carrying short unlabelled terminal segments (Fig. 7A, B). One pair was identified using FISH as the 4A chromosome, which is a well-known translocation (Naranjo et al. 1987, 1990; Chao et al. 1989; Liu et al. 1992; Devos et al. 1995). The second rearranged chromosome pair was further analysed by mcGISH using A- and D-genome probes and the translocation was demonstrated to be an A.D translocation (Fig. 10 A, B). Subsequently, FISH identified the rearranged chromosome as 7A, carrying a short terminal D-genome segment which could not be defined precisely by the pattern of the repetitive DNA probes (Fig. 6B, 8B). In addition, FISH revealed the absence of the 7D chromosome pair from the partial amphiploid. These observations suggested that the terminal D-genome segment on 7A could have arisen from the substituted 7D chromosome.

![Figure 10. A. GISH pattern of the 7A chromosome of line BE-1 using A-genome probe, with B and D genomes for blocking. The A genome is visualised in red. The short terminal unlabelled segment reveals an intergenomic rearrangement. B. GISH pattern of the 7A chromosome of BE-1 using D-genome probe and the AB genomes for blocking. The D genome is visualised in red. The red fluorescence on the terminal 7AL indicates that the translocated segment arose from the D genome.](image)

Twenty-four 7D-specific and eleven 7A-specific SSR markers were used in the present study in order to confirm the presence of the 7A.7D translocation chromosome and to describe its precise composition. Nine of the 35 markers tested showed no polymorphism between wheat cv. Bánkúti 1201 and the diploid genomes of *Th. bessarabicum* and *Ps. strigosa* used as control DNAs, so these results could not be used to characterise the translocation (Table 3).
Figure 11. **A.** Agarose gel electrophoresis pattern of the Xbarc111, Xbarc1046, Xbarc53, Xgwm428, Xgwm37, and Xcfd69 SSR markers specific to 7DL and of marker Xgdm142 with an unknown position on 7D on wheat cultivar ‘Bánkúti 1201’ (1), wheat–Th. ponticum partial amphiploid BE-1 (2), Th. bessarabicum (J genome) (3), and P. strigosa subsp. aegilopoides (S genome) (4). On the partial amphiploid and on ‘Bánkúti 1201’, fragments of the expected size were amplified by five primers (gdm142, barc53, gwm428, gwm37, and cfd69), proving the presence of 7DL, which carries these markers. **B.** Agarose gel electrophoresis pattern of the Xbarc29, Xcfa2123, Xcfa2257, Xgwm332, Xgwm63, Xgwm282, and Xbarc192 SSR markers specific to 7AL on ‘Bánkúti 1201’ (1), partial amphiploid BE-1 (2), Th. bessarabicum (3), and P. strigosa subsp. aegilopoides (4). The partial amphiploid lacked the fragments amplified by the gwm63, gwm282, and barc192 primers on ‘Bánkúti 1201’, proving the deletion of the minor segment of 7AL which carries these markers. **C.** Agarose gel electrophoresis pattern of the Xbarc126, Xbarc184, Xgwm111, and Xgwm44 SSR markers specific to 7DS on ‘Bánkúti 1201’ (1), partial amphiploid BE-1 (2), Th. bessarabicum (3), and P. strigosa subsp. aegilopoides (4). The partial amphiploid lacked the 7DS-specific bands amplified by these primers on ‘Bánkúti 1201’, indicating that 7DS was missing from the partial amphiploid.

Ten microsatellites mapped to 7DS were tested in the present work, including four markers that produced bands of the same size on the wheat and control species (Th. bessarabicum, Ps. strigosa) and were thus unsuitable to describe the translocation (Table 3).

Six of the markers (Xgdm130, Xwmc506, Xbarc126, Xbarc184, Xgwm111, Xgwm44) showed clear polymorphism between the wheat and control DNAs.
The partial amphiploid lacked the 7DS-specific fragments produced by primers gdm130, wmc506, barc126 and barc184 on wheat DNA. Xgwm44 is known to have two loci within the wheat genome, one on 4A and the other on 7DS. This primer pair amplified two fragments of different sizes (111bp, 182bp) on the parental wheat cultivar ‘Bánkútí 1201’, while on the amphiploid only the 111bp band specific to the 4A chromosome was amplified, the 182bp 7DS-specific fragment being missing (Fig. 11 C). As well as giving a 7DS-specific PCR product (208 bp) the gwm111 primer also amplified fragments of different sizes on the 2B and 7B chromosomes (132 bp, 150 bp) of ‘Bánkútí 1201’ wheat, but these fragments could be clearly differentiated from the 208bp 7DS-specific fragment. The partial amphiploid produced only the 2B- and 7B-specific bands and lacked the 208 bp 7DS-specific fragment (Table 3; Fig. 11 C). The absence of the 7DS-specific bands indicated that 7DS was missing from the partial amphiploid BE-1.

To test the presence of the 7DL chromosome arm, 14 SSR markers localized to the 7DL were used and polymorphism was detected in 11 cases between the wheat DNA and the control J- and S-genomic DNA (Table 3). The partial amphiploid BE-1 lacked the PCR products amplified on the parental wheat DNA by six 7DL-specific primers (gwm437, gdm46, gdm67, wmc94, barc111 and barc1046), indicating the absence of the 7DL region possessing these microsatellites (Table 3, Fig. 11 A).

However, four 7DL-specific primers (barc53, gwm428, gwm37, cfd69) and one primer with unknown marker position within the 7D chromosome (gdm142) gave PCR products of the expected size on both the wheat and amphiploid DNA, while the control J- and S-genomic DNA did not amplify these fragments (Fig. 11 A).

These results confirmed the presence of a short 7DL segment in the partial amphiploid BE-1. Markers mapped to the terminal regions of the 7A chromosome were used in order to reveal any deletion involving the distal region of the rearranged chromosome. Two markers mapped in the distal region of 7AS (Xbarc1088, Xbarc64) and four mapped on 7AL (Xbarc29, Xgwm332, Xcfa2123, Xcfa2257) produced PCR fragments of the expected size on both the wheat and amphiploid DNA (Fig. 11 B).

However, three of the tested 7AL-specific microsatellites (Xbarc192, Xgwm63, Xgwm282), mapped in the terminal region of 7AL, failed to give specific bands on the amphiploid DNA, revealing the elimination of a minor 7AL segment from the partial amphiploid BE-1 (Fig. 11 B).

These results confirmed the presence of a short chromosome segment derived from the eliminated 7D chromosome and revealed a minor terminal deletion involving 7AL. The
translocation breakpoint of the 7DL segment was located at FL 0.84 and the breakpoint interval (bin) can be demarcated by microsatellites Xgwm428, Xgdm142, Xcfd69, Xgwm37 and Xbarc53.
Table 3. Physical mapping of the 35 SSRs using the 7A.7D translocation of BE-1

Map name: Chinese Spring Deletion SSR
Ta-Synthetic/Opata-SSR_D-7D (0.00-267.90)
Wheat ABD-Wheat, Synthetic x Opata, BARC (0.00-149.20)
Wheat ABD-Wheat, Consensus SSR, 2004 (0.00-154.30)
Wheat ABD-Wheat, Composite, 2004 (0.00-241.00 cM)

Map Source: The GrainGenes 2.0 database (http://wheat.pw.usda.gov/)

* Marker data on the 7A.7D translocation of BE-1: 0: the marker showed no polymorphism between wheat and the diploid genomes of Th. bessarabicum and Ps. strigosa used as control DNAs; -: The marker was missing from the translocated chromosome; +: The marker was present on the translocated chromosome

<table>
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<th>Marker</th>
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<th>Map position within 7D</th>
<th>Annealing temperature (°C)</th>
<th>Marker data on the 7A.7D translocation of BE-1*</th>
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<td>0</td>
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<tr>
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<td>7DS, 2B</td>
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<td>147.90 cM</td>
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3.5 Hybridisation of the BE-1 partial amphiploid with wheat

Alien chromosomes in line BE-1 carry not only genes responsible for higher protein content and disease resistance but also many agronomically undesirable genes. These genes have to be eliminated with a series of backcrossing and selfing through several generations. Such a backcross program could result in progenies still having leaf rust resistance but carrying only a small segment from the *Th. ponticum* genome.

In order to decrease alien chromatin, line BE-1 was crossed with the Chinese Spring *ph* mutant (CS *ph*) wheat line under phytotron conditions. CS *ph* carries a mutation for the *Ph* gene, which results in induced homoeologous chromosome pairing in meiosis, so the frequency of intergenomic translocations increases in the progenies. Two approaches were used for the successful genetic transfer. In the first case, when BE-1 was used as female parent eight BE-1 spikes were emasculated and successfully pollinated using the CS *ph* mutant line (BE-1×CS *ph*). The second hybridisation experiment involved the reciprocal cross where nine spikes from CS *ph* mutant were emasculated and pollinated with BE-1 (CS *ph*×BE-1). Seed set was analysed in both cross combinations and is presented in Table 4. Higher seed set was found in the case of CS *ph*×BE-1, indicating that the wheat genotype used as female parent resulted in higher crossability. It should be noted that under phytotron conditions the pollen quantity was limited for of both cross combinations, which made pollination difficult. No embryo rescue was needed and both crosses resulted in well-developed seeds.

Seeds from the F₁ generation of both crosses were planted and grown in the Martonvásár field nursery in the season 2007-2008. All the progenies showed resistance to leaf rust compared to Mv9 kr1, which is a sensitive wheat line and showed extremely high leaf rust contamination. After emasculation three F₁ spikes originating from BE-1×CS *ph* were successfully backcrossed with Mv9 kr1 and seven other spikes with CS *ph*. Five F₁ spikes originating from CS *ph*×BE-1 were backcrossed with Mv9 kr1 wheat, while five other spikes were backcrossed with CS *ph* (Table 5).

The BC₁ progenies and the F₂ generation of BE-1×CS *ph* and CS *ph*×BE-1 were planted in the field nursery in the 2008-2009 season and their resistance to leaf rust was recorded. Plants originating from the backcross with Mv9 kr1 (which is a highly sensitive wheat line) showed different levels of resistance to leaf rust. Resistant plants from the F₂ generation were selected and planted in the nursery in 2009 October.
During the season 2008-2009 spikes of the F$_2$ progenies of the BE-1×CS $ph$ and CS $ph$×BE-1 crosses were pollinated with Mv Suba, a modern Martonvásár wheat cultivar. Crossability ranged from 16 to 32 % and in total 208 seeds were obtained from these crosses (Table 6). The BC$_1$ generation was planted in the Martonvásár field nursery in October 2009 in order to determine leaf rust resistance in the progenies. It is expected that stable progenies with 42 chromosomes will be obtained in the BC$_2$ generation, still carrying leaf rust resistance but with only a small segment from the alien genome. For this reason it is planned to analyse the chromosome composition of resistant plants from the BC$_2$ generation.

Seed set and the date of emasculation and pollination were recorded in each case (Tables 4, 5, 6.). Crossability (seed set percentage) was calculated by dividing the total number of seed set with the total number of florets pollinated and multiplying by 100.
Table 4. Seed set of the BE-1 partial amphiploid when pollinated with wheat line CS ph mutant and that of the reciprocal cross combination

<table>
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<tr>
<th>(plant code)</th>
<th>No. of florets pollinated</th>
<th>No. of seeds</th>
<th>Date of emasculation</th>
<th>Date of pollination</th>
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<tr>
<td>BE-1 (061423/1)</td>
<td>CSph</td>
<td>28</td>
<td>3</td>
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<td>BE-1 (061434/1)</td>
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<td>24</td>
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<tr>
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Σ=196 Σ=59 Crossability: 30%

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Σ=174 Σ=84 Crossability: 48.2%
Table 5. Seed set after backcrossing the BE-1 × CS \( ph \) and CS \( ph \) × BE-1 \( F_1 \) hybrids with different wheat genotypes (Mv9 kr1, CS \( ph \))

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<th>♂</th>
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</table>

Σ=402 Σ=139 Crossability : 34.5%

<p>| | | | | | | |</p>
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<td>32</td>
<td>0</td>
<td>14. 05. 2008</td>
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<td>7</td>
<td>17. 05. 2008</td>
<td>21. 05. 2008</td>
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<td>21. 05. 2008</td>
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<td>14</td>
<td>17. 05. 2008</td>
<td>21. 05. 2008</td>
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<td>Mv9kr(_1)</td>
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<td>21. 05. 2008</td>
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<td>42</td>
<td>23</td>
<td>17. 05. 2008</td>
<td>21. 05. 2008</td>
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</tr>
</tbody>
</table>

Σ=408 Σ=128 Crossability : 31.3%
Table 6. Seed set after backcrossing the BE-1 × CS ph and CS ph × BE-1 F$_2$ progenies with a modern Martonvásár wheat cultivar

<table>
<thead>
<tr>
<th>(plant code)</th>
<th>No. of florets pollinated</th>
<th>No. of seeds</th>
<th>Date of emasculation</th>
<th>Date of pollination</th>
</tr>
</thead>
<tbody>
<tr>
<td>(BE-1×CSph) izo (182/1-1)</td>
<td>MvSuba 28</td>
<td>11</td>
<td>15. 05. 2009</td>
<td>18. 05. 2009</td>
</tr>
<tr>
<td>(BE-1×CSph) izo (182/1-2)</td>
<td>MvSuba 28</td>
<td>0</td>
<td>15. 05. 2009</td>
<td>18. 05. 2009</td>
</tr>
<tr>
<td>(BE-1×CSph) izo (182/2)</td>
<td>MvSuba 26</td>
<td>7</td>
<td>15. 05. 2009</td>
<td>18. 05. 2009</td>
</tr>
<tr>
<td>(BE-1×CSph) izo (182/3)</td>
<td>MvSuba 28</td>
<td>5</td>
<td>15. 05. 2009</td>
<td>18. 05. 2009</td>
</tr>
<tr>
<td>(BE-1×CSph) izo (182/4)</td>
<td>MvSuba 30</td>
<td>6</td>
<td>15. 05. 2009</td>
<td>18. 05. 2009</td>
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<tr>
<td>(BE-1×CSph) izo (182/5-1)</td>
<td>MvSuba 24</td>
<td>0</td>
<td>15. 05. 2009</td>
<td>18. 05. 2009</td>
</tr>
<tr>
<td>(BE-1×CSph) izo (182/5-2)</td>
<td>MvSuba 20</td>
<td>2</td>
<td>15. 05. 2009</td>
<td>18. 05. 2009</td>
</tr>
</tbody>
</table>

| (CSph×BE-1) izo (183/1) | MvSuba 24 | 14 | 15. 05. 2009 | 18. 05. 2009 |
| (CSph×BE-1) izo (183/2-1) | MvSuba 24 | 12 | 15. 05. 2009 | 18. 05. 2009 |
| (CSph×BE-1) izo (183/2-2) | MvSuba 30 | 9 | 15. 05. 2009 | 18. 05. 2009 |
| (CSph×BE-1) izo (183/3-1) | MvSuba 28 | 2 | 15. 05. 2009 | 18. 05. 2009 |
| (CSph×BE-1) izo (183/3-2) | MvSuba 26 | 2 | 15. 05. 2009 | 18. 05. 2009 |
| (CSph×BE-1) izo (183/4-1) | MvSuba 30 | 11 | 15. 05. 2009 | 18. 05. 2009 |
| (CSph×BE-1) izo (183/4-2) | MvSuba 30 | 4 | 15. 05. 2009 | 18. 05. 2009 |
| (CSph×BE-1) izo (183/5) | MvSuba 28 | 15 | 15. 05. 2009 | 18. 05. 2009 |
| (CSph×BE-1) izo (183/6) | MvSuba 34 | 16 | 15. 05. 2009 | 18. 05. 2009 |
| (CSph×BE-1) izo (183/7) | MvSuba 32 | 0 | 15. 05. 2009 | 18. 05. 2009 |
| (CSph×BE-1) izo (183/8) | MvSuba 28 | 4 | 15. 05. 2009 | 18. 05. 2009 |
| (CSph×BE-1) izo (183/9) | MvSuba 26 | 15 | 15. 05. 2009 | 19. 05. 2009 |
| (CSph×BE-1) izo (183/10) | MvSuba 18 | 13 | 15. 05. 2009 | 19. 05. 2009 |

Σ=184 Σ=31 Crossability : 16.8%

Σ=358 Σ=117 Crossability : 32.6%
4. Discussion

Intergeneric hybrids between wheat and related species are generally almost completely sterile. The formation of amphiploids via the polyploidisation of such hybrids opens up the possibility to obtain fertile plants with a stable genetic background which includes the chromosome sets of both parents. Genome analysis showed clear evidence that the majority of angiosperm species have undergone polyploidisation, which was a major force in the dynamic evolution of plant genomes (reviewed by Wendel 2000). This process is known to induce major genome reorganisation including extensive gene loss and also changes in gene expression. In allopolyploids the rate of intergenomic translocations increases following the duplication event (Han et al. 2004; Feldman and Levy 2005). In general, chromosome aberration cause lethality in diploids while it can be widely supported in species carrying multiple genomes.

4.1 Chromosome composition of wheat-\textit{Th. ponticum} amphiploids

The wheat–\textit{Th. ponticum} partial amphiploid BE-1 was produced in the 1950s in Martonvásár and was used as genetic material for years, as it is a multiresistant (leaf rust, powdery mildew, personal communication, G. Vida) line with high protein content (20.8% protein and 49.9% gluten; personal communication, M. Rakszegi), though its chromosome composition remained unknown. The aim of this work was to reveal the detailed chromosome composition of BE-1, including intergenomic rearrangements, by means of ISH techniques (GISH, mcGISH, FISH) and SSR marker analysis.

Chromosome counting on the metaphase spreads after GISH revealed 56 chromosomes in the partial amphiploid. Both J and S genome probes detected 16 chromosomes derived from \textit{Th. ponticum} and 40 wheat chromosomes. The substituted wheat chromosome pair was identified by FISH as 7D. Among the alien chromosomes five pairs belonged unequivocally to the J genome, two pairs were J′ type chromosomes, while it was concluded that the remaining chromosome pair carried a segment originating from an unidentified genome in the pericentromeric regions.

Similar results were obtained by several authors, who reported the chromosome number and genome composition of other wheat-\textit{Th. ponticum} amphiploids using ISH with different genomic DNA probes. Chen et al. (1998b) detected 40 wheat and 16 alien chromosomes when applying GISH on three wheat–\textit{Th. ponticum} amphiploids (Agrotana,
OK7211542, ORRPX) using E, J and S genomic probes. The S-genome probe visualized eight J type chromosomes and eight J genome chromosomes in each of the amphiploids, but the substituted wheat chromosomes were not identified.

Fedak et al. (2000) reported the genomic composition of six wheat–Th. ponticum amphiploids (PMW706, PMW206, PMW209, PMWIII, OK7211542 and an Agropyron-wheat hybrid) revealed by GISH using S genomic DNA as a probe. The number of alien chromosomes varied from 12 to 18 among the amphiploids, but the chromosome number per cell was consistently 56. This suggested that chromosome substitutions occurred from the wheat genome, but the identity of the substituted chromosomes remained unknown. It was concluded that partial amphiploids originating from the same alien parent do not carry the same combination of alien chromosomes in all cases.

Li et al. (2003) analysed the chromosome composition of the leaf rust-resistant wheat–Th. ponticum amphiploid line 693 using labelled S genomic DNA isolated from Pseudoroegneria strigosa and detected 40 wheat chromosomes and 16 Th. ponticum chromosomes belonging to the J and Js genome, confirming that the alien genomes of Th. ponticum are present in a synthetic form and that both J and Js genomes are represented in the partial amphiploids.

Four other wheat–Th. ponticum amphiploids (SS5, SS156, SS363, SS660) were characterised by Oliver et al. (2006) using DNA from Th. ponticum as a probe. GISH detected 56 chromosomes per cell, but the number of chromosomes belonging to different genomes varied among the genetic materials. SS5 and SS156 carried 42 wheat and 14 alien chromosomes, while SS363 carried 40 wheat, 14 alien and 2 translocation chromosomes. SS660 carried 16 Th. ponticum and 40 wheat chromosomes. Based on meiotic pairing analysis in the F1 hybrids of these four amphiploids they were found to carry a similar set of Th. ponticum chromosomes, but the missing chromosomes and the chromosomes involved in the translocations were not identified.
4.2 J′ type chromosomes

Chen et al. (1998c) used GISH to define the genomic composition of *Th. ponticum* and concluded that *Th. ponticum* contains three sets of J genome and two sets of J′ genome chromosomes (JJJj′j). The J genome chromosomes were completely labelled when J genomic probe was added, while the J′ type chromosomes could only be detected using the S genome probe isolated from *Pseudoroegneria strigosa*, which hybridized to the centromeric and pericentromeric region of these chromosomes, while only the telomeres gave J genome-specific signals.

It should be noted that this type of mixed genome could be detected only in polyploid species of the *Thinopyrum* genus. Chen et al. (1998c) also demonstrated the presence of the J′ genome in the hexaploid *Th. intermedium* [(Host) Barkworth & D.R. Dewey], whose genomic composition was designated as J J′ S. Refoufi et al. (2001) reported the genomic composition of the hexaploid *Th. pycnantha* by GISH using J, E, S and P genome probes and demonstrated the presence of both P<sub>S</sub> and E<sub>S</sub> chromosomes, which were homologous to the P and J or E genomes, respectively, but showed strong affinity to the S genome probe in the pericentromeres, indicating the high homology of these areas to the S genome. Thus, the genomic formula of *Th. pycnantha* was proposed to be SSP<sub>S</sub>P<sub>S</sub>E<sub>S</sub>E<sub>S</sub>.

In the present study, after detecting *Th. ponticum* chromosomes with unlabelled pericentromeres using J genome probe an indirect method was used to detect chromosomes of the J′ type. In addition to the J genome probe, A or D genomic probes were used to demonstrate whether these unlabelled regions originated from either of the homoeologous genomes of hexaploid wheat (AD). Subsequently, GAA repetitive sequences were used (data not shown) to reveal if the pericentromeres arose from the B genome of wheat, as B-genome chromosomes show a specific banding pattern when these microsatellites are used as probes. McGISH and FISH using these probes confirmed that the alien chromosome segments in the centromeric region of the translocated chromosomes did not originate from the A, B or D genomes of wheat, though they may have had some similarity with the A and D genomes, as a faint fluorescent signal was observed when using these probes.

The S-genome probe showed only two chromosome pairs carrying signals characteristic of the J′ genome, while one chromosome pair carried unlabelled pericentromeres. The *Th. ponticum* probe (which included both the J and S genomes) still showed one chromosome pair with unlabelled pericentromeres, suggesting that this chromosome was different from those of the J′ type. It was concluded that as well as the J and
J's chromosomes, BE-1 carried one alien chromosome pair involved in a pericentromeric rearrangement, but the introgressed alien genome remained unidentified.

Centromeres are known to play an important part in correct chromosome segregation during cell division, but little is known about the function of the pericentromeric regions (Guyot et al. 2005). In Arabidopsis (Arabidopsis thaliana) and rice (Oryza sativa), the centromeres are mainly composed of specific tandemly repeated satellite DNA, occasionally interrupted by the insertion of long terminal repeat (LTR) retrotransposons (Copenhaver et al., 1999; Wu et al., 2004; Zhang et al., 2004), while the pericentromeric regions are rich in moderately repetitive elements, including transposons, retrotransposons, and pseudogenes (Copenhaver et al., 1999). In addition, more than 200 expressed genes were discovered in the pericentromeric regions of the Arabidopsis genome (Copenhaver et al., 1999), indicating that transcription is not influenced by the heterochromatic environment.

Shotgun and BAC-end sequencing has demonstrated that the human centromere consists of α-satellite DNA, a tandemly repeated 171 bp sequence, blocks of highly repetitive heterochromatin and LINE-type retroelements, as well as other sequences (Schueler et al. 2001).

Major structural rearrangements are often connected with heterochromatic regions and translocation breakpoints frequently occur at the heterochromatic-euchromatic borders, so centromeres and pericentromeres are hotspots for such rearrangements (Badaeva 2007, Raskina 2008).

Large rearrangements have been detected in the pericentromeres of A. thaliana ecotypes, including the insertion of tandem copies from the mitochondrial genome to pericentromere II (Stupar et al. 2001), and a large inversion involving pericentromere IV, which was found to be specific to several ecotypes (Fransz et al. 2000). Rapid karyotypic changes have been associated with the activity of mobile elements. The host defence model demonstrated that during evolution mobile element activity was repressed by DNA methylation (Bestor and Tycko 1996; Yoder et al. 1997). In cultured human cells the use of DNA methylation inhibitors significantly increased the frequency of rearrangements in the pericentromeric regions of Chr1 and at lower frequencies in the pericentromeres of Chr16, suggesting that DNA demethylation causes chromosome instability (Hernandez et al. 1997, Ji et al. 1997).

The origin of the J' genome is still to be discovered but all these studies point to the fact that hybridisation and polyploidisation cause major genome reorganisation and the centromeric chromatin is extremely sensible to such rearrangements. Th. ponticum is a
segmental allopolyploid with an extremely high ploidy level, thus representing an intermediate state between the auto- and allopolyploid species. During its speciation it underwent on a number of evolutionary processes such as whole genome duplication (autopolyploid character) and interspecific hybridisation (allopolyploid character). In conclusion, the appearance of the \( J' \) genome, retaining a large part of the \( S \) genome in the pericentromeres, could be the result of complex genetic and epigenetic changes generated by ancient polyploidisation and hybridisation events.
4.3 Physical mapping using mcGISH and SSR markers

Using GISH and mcGISH, Han et al. (2004) characterised the genomic composition of five wheat–Th. intermedium amphiploids classified into two types (type I: Zhong 1 and Zhong 2, type II: Zhong 3 and Zhong 5). Although the amphiploids belonged to the same type and were derived from the same backcross pedigrees, different types of genomic rearrangements were detected. The gliadin and HMW glutenin patterns of this plant material showed novel expression patterns compared to the parental progeny, showing that allopolyploidisation accelerates genomic changes in wheat.

Fedak and Han (2005) described the mcGISH pattern of addition lines (2n = 44) derived from the wheat–Th. intermedium amphiploids TAF 46 and Zhong 5 and detected rearrangements involving the A, B and D genomes of wheat and the genomes of Th. intermedium.

A similar approach was used in the present study to reveal intergenomic rearrangements between the A, B and D genomes of wheat and the genomes arising from the Thinopyrum parent. Although the mcGISH data provided no evidence for rearrangements between the wheat and Thinopyrum chromosomes, a new translocation was identified by mcGISH and FISH. The absence of the 7D chromosome pair suggested that the translocation involved 7AL and a short 7D chromosome segment. SSR marker analysis confirmed the presence of the 7A.7D translocation and also demonstrated that the translocation could be useful in the physical mapping of 7DL and 7AL.

Three of the markers proving the presence of the short 7DL segment (Xgwm37, Xcfd69, Xbarc53) were selected from a previously published deletion map, where they were mapped to the terminal breakpoint interval (bin) of 7DL, between FL 0.82 and 1.00 (Sourdille et al. 2004) (Table 3; Fig. 12 A). Two other markers (Xbarc111, Xbarc1046) were also mapped physically to the terminal bin of 7DL (FL 0.82–1.00) (Sourdille et al. 2004), but these markers were absent from the translocated segment, suggesting that the translocation breakpoint could be placed distally to that of the deletion lines used in establishing the previous physical maps (Figs. 11A, B). Fine mapping of the 7AL.7DL translocation will provide a new physical landmark on chromosome arm 7DL, facilitating more precise physical mapping of the terminal region of 7DL.

Discrepancies were found in the positions of some markers compared with previous physical mapping studies. Among the markers present on the translocated 7DL segment, Xgwm37, Xcfd69, and Xbarc53 were mapped previously to the terminal bin of 7DL between
FL 0.82 and 1.00, while Xgwm428 was positioned in a proximal bin between FL 0.30 and 0.61 (Sourdille et al. 2004) (Fig. 12 A). In the present study, Xgwm428 was localized distally to Xbarc111 and Xbarc1046 (Fig. 12 B), both of which were mapped previously to the terminal bin of 7DL (Fig. 12 A). The inverted order of loci Xbarc111, Xbarc1046, and Xgwm428 can be explained by the fact that the deletion lines used for the previous physical mapping analysis were derived from the wheat cultivar ‘Chinese Spring’ (Endo and Gill 1996), while the present translocation was detected in the genetic background of the wheat cultivar ‘Bánkúti 1201’. A paracentric inversion occurring in one of the parental cultivars prior to the deletion event could explain the reversion in the marker order. Similar discrepancies have been found by other authors.

**Figure 12.** A. Localisation of 7DL-specific markers on the deletion bins of deletion lines derived from Chinese Spring, the wheat cultivar used in the previous physical mapping of 7DL. The deletion breakpoints are indicated by lines and the fraction lengths are indicated on the left of the schematic chromosome. The positions of the markers are indicated by arrows. B. Physical mapping of the tested 7DL-specific markers on the terminal 7DL segment identified in the present study. The positions of the markers are indicated by arrows. The marker whose position was previously unknown and which was mapped physically to the terminal region of 7DL is shown in red. The fraction length is indicated on the right side of the chromosome. C. GISH pattern of the 7AL.7DL translocation chromosome using A- and D-genome probes. The short D-genome segment is visualized in green and the A genome in red. D. Physical mapping of the tested 7AL-specific markers using the terminal deletion detected in the present study. The positions of the markers are indicated by arrows. The marker whose precise position was previously unknown within fraction length 0.39–1.00 and which was mapped physically to the 7AL terminal region is shown in red. E. Localisation of 7AL-specific markers on the deletion bins of deletion lines derived from Chinese Spring, the wheat cultivar used in the previous physical mapping of 7AL. The deletion breakpoints are indicated by lines and the fraction lengths are indicated on the right of the schematic chromosome. The positions of the markers are indicated by arrows.
During the construction of a physical map for the homoeologous group 7 chromosomes of common wheat, Werner et al. (1992) detected a reversion in the order of RFLP markers Xpsr72 and Xpsr169 and explained the reversion by an inversion during the production of the deletion line.

Chen and Gustafson (1995) detected a change in the physical location of the RFLP marker Xpsr117 and the α-amylase locus on chromosomes 7B and 7D compared with previously published genetic maps. It was concluded that the physically mapped restriction fragments were different from those used in the construction of the genetic maps, both markers being multilocus family clones. However, the map positions of the 7DL-specific microsatellites revealed in the present study correlated well with other reference maps. The genetic map derived from a cross between the synthetic wheat ‘W7984’ and ‘Opata’ placed Xgwm428 distally to Xbarc111 (Song et al. 2005, Table 3). Good agreement was also found with the wheat microsatellite consensus map published by Somers et al. (2004), which placed Xgwm428 in the terminal region of 7DL, distally to Xbarc111.

The marker Xgdm142 was previously described on chromosome 7D by Pestsova et al. (2000), although its precise position within the chromosome was unknown. The present analysis placed Xgdm142 on the translocated 7DL segment demarcated by four 7DL-specific microsatellites (Figs. 12B, C), demonstrating that the 7AL.7DL translocation chromosome can be used to physically map SSR markers or genes to the terminal region of 7DL.

A minor deletion involving the distal region of 7AL was detected by 3 microsatellites (Xbarc192, Xgwm63, Xgwm282) (Fig. 12D). Previously, 2 of these markers (Xgwm63, Xgwm282) were mapped physically to the 0.86–0.90 bin (Fig. 12E). The third marker was designed for the interval 0.39–1.00, covering 4 bins, without a precise position, so the present study placed it unequivocally in the terminal region of 7AL (Fig. 12D). The deletion breakpoint can be placed between markers Xgwm332 and Xgwm63, both mapped previously to the 0.86–0.90 bin (Figs. 12D, E). The terminal deletion involving 7AL suggests that a chromosome break occurred in the terminal region of 7AL during the translocation event, followed by the loss of the short segment. The breakage on 7AL fused with a short telomeric 7DL segment derived from a similar breakage involving the distal region of 7DL. The rearrangement resulted in the elimination of the proximal region of 7DL and the whole of 7DS.

Differences between genetic and deletion mapping studies of common wheat may be due to the fact that genetic maps are based on the recombination frequency between markers, which varies along the chromosome, thus providing only an estimate of the order and the
genetic distances. Recombination hotspots usually occur in gene-rich regions, and crossovers are strongly suppressed in the heterochromatic regions characteristic of the centromeres (Gustafson et al. 1990; Lukaszewski and Curtis 1993; Peters et al. 2003). Moreover, the present study revealed that the position of markers may vary from one wheat cultivar to the other, so the use of a larger sample of aneuploid wheat lines produced from diverse cultivars would make more detailed mapping analysis possible.

The present study demonstrated that in situ hybridisation techniques, combined with SSR marker analysis, are extremely useful in detecting and identifying intergenomic rearrangements in the wheat genome, leading to the selection of genetic materials useful for future mapping studies. The fine mapping of the 7AL.7DL translocation chromosome opens up the possibility of more precise physical mapping of the terminal regions of 7DL and 7AL.

Knowledge on the cytogenetic background of the genetic material used in wheat improvement is crucial for designing modern breeding programs. The exploration of the intergenomic rearrangements occurring in wheat-alien amphiploids using in situ hybridisation and molecular markers will facilitate the selection of progenies carrying the chromosome segments associated with agronomically important genes. Such studies also provide a better understanding of the process of alien introgression, revealing the effects of various introgressions and rearrangements in the genetic background of wheat.
5. **New scientific results**

- The validation of the hybrid status of the leaf rust-resistant, high-protein partial amphiploid BE-1 using GISH. All chromosomes originating from the *Th.* parent were visualised and differentiated from each other by combining GISH and FISH results.
- All the wheat chromosomes present were individually identified and the elimination of a major 7D chromosome segment was detected by mcGISH, FISH (using repetitive DNA probes) and SSRs.
- mcGISH detected a new translocation occurring between the A and D genomes of wheat.
- mcGISH and SSR markers together identified the fine composition of the translocated chromosome as 7AL.7DL.
- The new translocation was used to physically map the 7D-specific SSR marker Xgdm142 to the terminal region of 7DL.
- New deletion breakpoints were discovered within 7DL and 7AL, providing new physical landmarks within these regions.
- A wheat-alien amphiploid was used for the first time for the physical mapping of SSRs.

6. **Conclusions and prospects**

Due to their agronomically important traits, fertility and crossability, wheat-alien amphiploids are ideal genetic material for wheat improvement. The detailed cytological analysis of the partial amphiploid BE-1 reported in the present study using different *in situ* hybridisation methods (GISH, mcGISH, FISH with repetitive DNA probes) enables the transfer of *Th. ponticum* chromosomes from this amphiploid into wheat to be traced. As a good source for improving disease resistance and quality, BE-1 could be a promising crossing partner in wheat breeding programs. The BE-1 partial amphiploid was crossed with the *ph* mutant line of the wheat cultivar CS and the progenies were subsequently crossed with wheat line Mv9 *kr1* and the Martonvásár wheat cultivar Mv Suba. Cytologically stable, resistant hybrid plants selected from the progenies can be used as leaf rust resistance sources in the wheat breeding crossing program in Martonvásár.
In addition, a combination of mcGISH and chromosome-specific SSR markers demonstrated that partial amphiploids carrying different intergenomic rearrangements can be used as model organisms for the physical mapping of molecular markers to a precise chromosomal region. The combination of these techniques revealed an intergenomic rearrangement between the wheat genomes, which was precisely identified as a 7AL.7DL translocation. The determination of new translocation breakpoints and their positions opened up the possibility of physically mapping molecular markers to the terminal regions of 7AL and 7DL.

7. Acknowledgements

This work would not have been possible without the professional support and encouragement of my supervisor Dr. Márta Molnár-Láng. Particular thanks go to my colleague Dr. István Molnár for his fruitful ideas and for the detailed discussions of numerous technical problems. I owe many thanks to Mrs. Julianna Bucsi and Mrs. Emilia Havasi for their technical assistance, whenever I needed it and of course for their sense of humour especially on Friday afternoons. I am also indebted to Barbara Harasztos for revising the manuscript linguistically.
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9. Scientific publications

9.1 Peer-reviewed scientific papers:


9.2 Conference proceedings:


Summary

In situ hybridisation (mcGISH and FISH) was used to characterise the genomic composition of the wheat–Thinopyrum ponticum partial amphiploid BE-1. The amphiploid is a high-protein line having resistance to leaf rust (Puccinia triticina) and powdery mildew (Blumeria graminis f. sp. tritici) and has a total of 56 chromosomes per cell. GISH using the J and S genomes and Th. ponticum DNA as probes showed 16 chromosomes originating from Thinopyrum ponticum. Multicolour GISH using J, A and D genomic probes revealed not only the 16 Th. chromosomes but also 14 A-genome, 14 B-genome and 12 D-genome chromosomes, indicating that one pair of D chromosomes has been replaced by an alien chromosome pair. FISH using Afa family, pSc119.2 and pTa71 probes allowed the identification of all the wheat chromosomes present, and the missing chromosome pair was identified as 7D.

Six of the Th. ponticum chromosomes carried segments different from the J genome in their centromeric regions. Four of these chromosomes have been demonstrated to be J’ type chromosomes carrying segments similar to the S genome near the centromeres. It was also demonstrated that the alien chromosome segments in the pericentromeric region of the remaining rearranged Th. chromosomes did not originate from the A, B or D genomes of wheat, so this translocation remains to be identified. The localisation of the repetitive DNA sequences Afa family, pSc119.2 and pTa71 on the Th. ponticum chromosomes of the amphiploid was described for the first time in the present study. On the basis of their multicolour FISH patterns, the alien chromosomes could be arranged in eight pairs and could also be differentiated unequivocally from each other, making it possible to trace the alien chromosome segments in the progenies.

Rearrangement was detected between the A and D genomes of wheat and was precisely identified as 7AL.7DL. In both cases (7AL and 7DL) the position of the translocation breakpoint was different from that of known deletion lines. The identification of the 7AL.7DL translocation and its breakpoint position provides a new physical landmark for future physical mapping studies, opening up the possibility of more precise localisation of genes or molecular markers within the terminal regions of 7DL and 7AL.

The partial amphiploid has been crossed with various wheat lines and with a Martonvásár wheat cultivar and fertile hybrid plants have been obtained. Resistant progenies can be used as an important source of leaf rust resistance in wheat breeding programs.
Az in situ hibridizáció módszerét (GISH, mcGISH és FISH) alkalmaztuk a BE-1, búza- Thinopyrum ponticum részleges amfiploid részletes citogenetikai vizsgálatához. Az amfiploid magas fehérjetartalmú és rezisztens levélrozsdával (Puccinia triticina) és lisztharmattal (Blumeria graminis f. sp. tritici) szemben. Sejtenként 56 kromoszómát tartalmaz. GISH J-, S genomi és Th. ponticum teljes genomi próba felhasználásával 16 idegen kromoszómát mutatott ki. Multikolor genomi in situ hibridizáció J genomi és A illetve D genomi próba egyidejű hibridizációja során a 16 idegen kromoszóma mellett 14 A genom, 14 B genom és 12 D genom kromoszómát detektált, jelezve, hogy egy D kromoszóma pár kieset és azt egy pár idegen kromoszóma helyettesíti. FISH Afa family, pSc119.2 és pTa71 repetitív próbák felhasználásával kimutatta, hogy a hiányzó kromoszóma pár a 7D.


Átrendeződést figyeltünk meg a búza A és D genomjai között és a transzlokációt SSR markerek segítségével 7AL.7DL-ként azonosítottuk. Mindkét esetben (7AL, 7DL) a transzlokációs töréspontok helyzete eltért az eddig leírt deléciós vonalak töréspontjaitól. Az új 7AL.7DL transzlokáció új fizikai határjelzőként szolgál a későbbi fizikai térképezésben lehetővé téve a markerek helyzetének még pontosabb meghatározását a 7AL és 7DL terminál régióban.

A BE-1 részleges amfiploidot fitotroni körülmények között kereszteztem CS ph mutáns búzavonallal. A hibrideket tenyészkerti körülmények között visszakereszteztünk az Mv9 kr1 búzavonallal és az Mv Suba búzafajtával. Az utodók közül tervezem citológiaiag stabil, rezisztens növények kiválogatását amelyek új, rezisztens genetikai alapanyagként felhasználhatóak a búzanemesítésben.